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Potential Diarrheal Pathogens Common Also in Healthy Children in Angola

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Background: Globally, diarrhea kills almost 1500 children daily. In diagnostics, molecular methods are replacing traditional assays. We aimed to investigate enteropathogens in children with and without diarrhea in Luanda, the capital of Angola.

Methods: One hundred and ninety-four stool samples from 98 children with acute diarrhea and 96 children without diarrhea were investigated for 17 enteropathogens with multiplex real-time polymerase chain reaction.

Results: The median age of children was 10.5 months. Enteropathogens, bacteria, viruses and parasites were detected in 91%, 78%, 50% and 25%, respectively. A positive finding was significantly ($P = 0.003$) more common in diarrhea when testing for all pathogens combined, for bacteria alone and for viruses alone. More than one pathogen was found more frequently in diarrhea than in non-diarrhea stool samples, in 87% and in 59% ($P < 0.0001$), respectively. The median number (interquartile range) of pathogens detected was 3 (2) versus 1.5 (2; $P < 0.0001$), respectively. When age was taken into account, diarrhea was found to be associated with enterotoxigenic and enteroaggregative *Escherichia coli*, *Shigella*, *Campylobacter*, rotavirus, sapovirus and *Cryptosporidium*.

Conclusions: Multiplex polymerase chain reaction detected enteropathogens in almost all stool samples of children in Luanda, albeit this occurred more often in diarrhea. Children with diarrhea showed more mixed infections than children without diarrhea.

Key Words: diarrhea, children, developing countries, etiology, multiplex real-time PCR

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Globally, diarrhea causes annually more than 500,000 deaths in children under 5 years old.¹ Even though diarrhea mortality has reduced >30% in 2000–2015,¹ the burden of the disease is still enormous in low- and middle-income countries.² Here, the children under 5 years old experienced 1.9 billion and 1.7 billion episodes of diarrhea in 1990 and 2010, respectively.² Knowing the causative agent is important for epidemiologic surveillance, for planning of preventive measures such as vaccines, and sometimes, for a specific antimicrobial treatment.

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In the developing world, the etiology of diarrhea has been searched for mostly by using traditional assays.^{3–13} Some of these studies have included only children with diarrhea without a control group.^{3,5,7,9} In a recent review, rotavirus, calicivirus, enteropathogenic and enterotoxigenic *Escherichia coli* were estimated to cause more than half of all diarrheal deaths in children under 5 years old.¹⁴ Global Enteric Multicenter Study (GEMS) found rotavirus, *Cryptosporidium*, enterotoxigenic *E. coli* and *Shigella* to be responsible for most cases of moderate-to-severe diarrhea.¹¹ However, the importance of the pathogens varied according to the study site.¹¹ A multisite birth cohort study (MAL-ED) concluded that there prevailed substantial heterogeneity in terms of the child's age, geography, season, rotavirus vaccine status and symptoms.¹²

Obviously, methods with a wider capability to detect various agents must be used.^{15–17} On the other hand, antibiotics, which are recommended only in bloody diarrhea or severe cholera, are still frequently used and may render bacterial cultures negative.¹⁸ Fortunately, recent years have brought various multiplexed molecular assays, which target enteric pathogens directly from fecal specimens.^{16,19,20} Multiplex polymerase chain reaction (PCR) is another step forwards because it detects pathogens remaining unidentified by many conventional methods.^{15,21} Five different laboratories used these tests for 15 common enteropathogens and found high sensitivity and good analytical and clinical performance also in childhood diarrhea.¹⁷ Often, multiplex PCR reveals polymicrobial infections in diarrhea patients.^{13,22,23} As enteropathogens are often found also in children without diarrhea, quantitative analysis may help distinguish clinically significant infections.^{13,17,24,25} A reanalysis of the GEMS study using quantitative molecular methods improved characterization of the causes of diarrhea and indicated *Shigella* or enteroinvasive *E. coli*, rotavirus, adenovirus, enterotoxigenic *E. coli*, *Cryptosporidium* and *Campylobacter* as most important enteropathogens.²⁶

Angola, a country in sub-Saharan Africa, has the highest reported under-5 mortality in the world, with 156.9 deaths per 1000 live births.²⁷ It is also one of the countries with highest burden of diarrhea in children under 5 years old, with estimated 3.5 episodes per child-year and 18,000 annual deaths.²⁸ We aimed to investigate enteropathogens with multiplex PCR in children with and without diarrhea in Luanda, the capital of Angola.

MATERIALS AND METHODS

Material

The Pediatric Hospital of Luanda is a tertiary teaching and referral hospital. Most patients are self-referred and arrive from the greater metropolitan area. This study, approved by the institution's Ethics Committee, was carried out at the emergency department, which examines about 300 children each day; approximately 15% of these present with diarrhea. The study included children under 5 years of age whose diarrhea had lasted less than 2 weeks, who attended the Hospital in December 25, 2013, to August 7, 2014, and of whom a stool sample could be collected.

Diarrhea was defined as three or more loose or liquid stools or at least one bloody stool a day. The control stool samples were collected during May 16 to October 5, 2014, from children with no diarrhea who

visited the institution for vaccinations, physiotherapy or a surgeon's consultation. Rotavirus vaccination was implemented in Luanda in the end of 2014 only after our study. ESwab (Copan, Italy) liquid-based collection and transport system was used, and the samples were frozen in -70°C the day of collection. The samples were transported frozen to Finland for multiplex PCR. Children were considered malnourished if their weight-for-height was less than -2 Z score.

Laboratory Methods

Samples and DNA Extraction

The stool samples in ESwab tube were extracted with NucliSENS EasyMAG (bioMérieux, France) according to the manufacturer's protocol for Generic 2.0.1 program. For extraction of bacteria and viral nucleic acids, 70–100 μL of stool sample was added directly to the 2 mL of lysis buffer for off-board lysis step. Additional pretreatment protocol was executed for parasites. Shortly, 200 μL of stool samples and 1 mL of lysis buffer was added to the MagNA Lyser Green Beads (Roche, Switzerland) tube, and the samples were bead beaten in MagNA Lyser (Roche, Switzerland) instrument twice at 7000 oscillations per minute for 60 s having 90 s intermediate cooling steps after each bead-beating step. Samples were centrifuged at 10,000g for 5 min, and supernatant together with 1 mL of lysis buffer was transferred to the off-board lysis steps. Automated extraction in NucliSENS EasyMAG was executed for all lysed samples using 100 μL as elution volume.

Real-Time PCR and Analysis

Samples were analyzed with 3 different real-time PCR assays. The Amplidiag Bacterial GE, Amplidiag Viral GE and Amplidiag Stool Parasites (Mobidiag, Finland) assays are developed for identification of various gastroenteritis-causing bacteria, viruses and parasites and are based on multiplexed real-time PCR amplification in conjunction with fluorophore-labeled probes. The PCR reactions were conducted according to the instructions of the each assays. Internal amplification control, positive PCR control and negative PCR control are included to the each test series. The PCR runs for bacteria and parasites were carried out in the Bio-Rad CFX qPCR Systems and CFX Manager Software (Bio-Rad, Hercules, CA). The PCR run for viruses was carried out in the ABI7500Fast real-time PCR instrument and ABI7500Fast Software (Applied Biosystems, Waltham, MA). Run files were exported from the PCR software to the Amplidiag Analyzer (Mobidiag, Finland), which analyses and reports bacterial, viral and parasitic findings from each sample automatically. Semiquantitative estimates of pathogen load were analyzed based on threshold cycle (C_t) values.

Statistical Analysis

All data were computed and analyzed with Statview 5.1. The statistical tests comprised Fisher's exact, χ^2 , Student t test,

Mann–Whitney U test, Kruskal–Wallis, Spearman correlation and logistic regression, as appropriate. Statistical significance was defined as P value below 0.05.

RESULTS

The analysis included 194 children less than 60 months of age, 98 children with and 96 without diarrhea. The median age was 10.5 months [interquartile range (IQR), 17] overall, 8 months (IQR, 7) for children with and 18 months (IQR, 21) for those without diarrhea ($P < 0.0001$); 103 children were female and 91 male ($P = 0.57$).

Of the children with diarrhea, 30 were 0–5 month old, 44 were 6–11 months old and 24 were 12–59 months old. Of them, 72% (70 of 97) showed also vomiting, 76% (71 of 94) fever and 59% (57 of 96) mucus in stools. Nineteen children complained abdominal pain and 5 had blood in stools. Likely diarrhea-caused dehydration was found in 57% (54 of 94) of children. Of children with diarrhea, 85% (79 of 93) had consulted before, 76% (71 of 94) had received some medication and 60% (52 of 87) also antibiotic treatment.

Running water at home was lacking more often in the diarrhea than in the non-diarrhea group (23 of 80, 29% vs. 39 of 87, 45%, $P = 0.032$). A tendency for less diarrhea was observed for children who used bottled or filtered drinking water and who had a toilet at home (Table 1). Malnourishment was more common in the diarrhea than in the non-diarrhea group, 45% (44 of 97) versus 17% (16 of 94, $P < 0.0001$).

Of the stool samples, enteropathogens overall and bacteria, viruses and parasites were detected in 176 (91%), 152 (78%), 97 (50%) and 48 (25%), respectively (Table 2). When comparing the diarrhea versus the non-diarrhea groups, positive samples were found significantly more often in the former group when testing for all pathogens combined (95 vs. 81, $P = 0.003$), for bacteria alone (86 vs. 66, $P = 0.001$) and for viruses alone (64 vs. 33, $P < 0.0001$; Table 2). In contrast, no such difference was found for parasites (28 vs. 20, $P = 0.21$). Mixed infections with more than one pathogen group (bacteria, viruses or parasites) were found in 101 of 176 (57%) of all positive samples, in 67 of 95 (71%) of children with and in 34 of 81 (42%) of those without diarrhea ($P = 0.0001$). Furthermore, more than one pathogen was found in 131 of 176 (74%) of all positive samples and in 83 of 95 (87%) of children with and in 48 of 81 (59%) of those without diarrhea ($P < 0.0001$). In the diarrhea versus the non-diarrhea groups, the median number (IQR) of pathogens detected was 3 (2) versus 1.5 (2; $P < 0.0001$); for bacteria, the numbers were 2 (2) versus 1 (2; $P < 0.0001$); for viruses, 1 (1) versus 0 (1; $P < 0.0001$); and for parasites, 0 (1) versus 0 (0; $P = 0.24$).

Regarding the children showing a positive PCR (of any kind), there was an association with age ≥ 6 months ($P = 0.002$),

TABLE 1. Characteristics of Children With and Without Diarrhea

Variable	N Examined	All Children	Children With Diarrhea	Children Without Diarrhea	P
Age (months), median (IQR)	194	10.5 (17)	8 (7)	18 (21)	<0.0001
Female	194	103 (53%)	54 (55%)	49 (51%)	0.57
WFA* Z score	189	−1.0 (1.9)	−1.4 (1.9)	−0.4 (1.7)	<0.0001
Malnutrition	191	60/191 (31%)	44/97 (45%)	16/94 (17%)	<0.0001
Number of people at home, median (IQR)	186	5 (3)	6 (3)	5 (2.5)	0.034
Number of children at home, median (IQR)	186	3 (3)	3 (2)	2 (3)	0.005
Running water at home	167	62/167 (37%)	23/80 (29%)	39/87 (45%)	0.032
Filtered or bottled drinking water	159	35/159 (22%)	12/77 (16%)	23/82 (28%)	0.058
WC† or latrine at home	176	146/176 (83%)	72/92 (78%)	74/84 (88%)	0.080

*Weight-for-age.

†Water closet.

TABLE 2. Results of Multiplex PCR of Stool Samples of Children With and Without Diarrhea

Variable	N Examined	All Children	Children With Diarrhea	Children Without Diarrhea	P
		194	98	96	
Positive PCR for any pathogen	194	176 (91%)	95 (97%)	81 (84%)	0.003
Positive PCR for bacteria	194	152 (78%)	86 (88%)	66 (69%)	0.001
Number of pathogens detected, median (IQR)	194	2 (2)	3 (2)	1.5 (2)	<0.0001
>1 pathogen detected	176	131/176 (74%)	83/95 (87%)	48/81 (59%)	<0.0001
≥2 group vs. 1 group of pathogens detected	176	101/176 (57%)	67/95 (71%)	34/81 (42%)	0.0001
Number of bacteria in the stool sample	194	1 (1)	2 (2)	1 (2)	<0.0001
Enteropathogenic <i>Escherichia coli</i>	194	100 (52%)	52 (53%)	48 (50%)	0.67
Enterotoxigenic <i>Escherichia coli</i>	194	54 (28%)	40 (41%)	14 (15%)	<0.0001
Enterotoxigenic <i>Escherichia coli</i>	194	89 (46%)	57 (58%)	32 (33%)	0.0005
<i>Shigella</i> /Enteroinvasive <i>Escherichia coli</i>	194	29 (15%)	18 (18%)	11 (11%)	0.18
<i>Campylobacter</i>	194	29 (15%)	23 (23%)	6 (6%)	0.0008
<i>Salmonella</i>	194	2 (1%)	2 (2%)	0 (0%)	0.50
Positive PCR for viruses	194	97 (50%)	64 (65%)	33 (34%)	<0.0001
Number of viruses in the stool sample	194	0.5 (1)	1 (1)	0 (1)	<0.0001
Rotavirus	194	40 (21%)	30 (31%)	10 (10%)	0.0005
Adenovirus	194	7 (4%)	5 (5%)	2 (2%)	0.44
Norovirus	194	43 (22%)	26 (27%)	17 (18%)	0.14
Astrovirus	194	7 (4%)	3 (3%)	4 (4%)	0.72
Sapovirus	194	22 (11%)	19 (19%)	3 (3%)	0.0004
Positive PCR for parasites	194	48 (25%)	28 (29%)	20 (21%)	0.21
Number of parasites in the stool sample	194	0 (0)	0 (1)	0 (0)	0.24
<i>Giardia</i>	194	27 (14%)	12 (12%)	15 (16%)	0.50
<i>Cryptosporidium</i>	194	22 (11%)	19 (19%)	3 (3%)	0.0004
<i>Dientamoeba</i>	194	7 (4%)	1 (1%)	6 (6%)	0.063

not being exclusively breastfed ($P = 0.036$) and eating solid foods ($P = 0.036$). A positive PCR for bacteria alone related also with age ≥ 6 months ($P = 0.002$), not being exclusively breastfed ($P = 0.004$) and eating solid foods ($P = 0.002$), whereas a positive PCR for viruses alone associated only with age < 12 months ($P = 0.003$). Interestingly, no clear associations were found for parasites. The number of bacteria had a weak negative correlation with the child's weight for age Z score (Rho corrected for ties -0.155 ; $P = 0.034$). In the diarrhea group, the age correlated with the number of detected pathogen groups (Rho corrected for ties, 0.285 ; $P = 0.005$), with the number of detected pathogens (Rho corrected for ties, 0.375 ; $P = 0.0002$), with the number of bacteria (Rho, 0.293 ; $P = 0.004$) and with the number of parasites (Rho, 0.213 ; $P = 0.036$), but not with the number of viruses. In all children, this kind of correlation with age, albeit negative, was found only for viruses (Rho, -0.152 ; $P = 0.035$).

Enteropathogenic *E. coli* was detected in 100 (of 194, 52%) samples, enterotoxigenic *E. coli* in 54 (28%), enteroaggregative *E. coli* in 89 (46%), enteroinvasive *E. coli*/*Shigella* in 29 (15%), *Campylobacter* in 29 (15%) and *Salmonella* in 2 (1%) cases (Table 2). Of viruses, PCR for rotavirus was positive in 40 (21%), for adenovirus in 7 (4%), for norovirus in 43 (22%), for astrovirus in 7 (4%) and for sapovirus in 22 (11%) samples. Of parasites, *Giardia* was found in 27 (14%), *Cryptosporidium* in 22 (11%) and *Dientamoeba* in 7 (4%) samples.

Diarrhea was found to be associated with the detection of enterotoxigenic ($P < 0.0001$) and enteroaggregative ($P = 0.0005$) *E. coli*, *Campylobacter* ($P = 0.0008$), rotavirus ($P = 0.0005$), sapovirus ($P = 0.0004$) and *Cryptosporidium* ($P = 0.0004$; Table 2). When age as a confounding variable was taken into account, odds ratio for diarrhea with enterotoxigenic *E. coli* was 3.95 [95% confidence interval (CI): 1.84–8.48; $P = 0.0004$]; for enteroaggregative *E. coli*, 2.21 (95% CI: 1.16–4.22; $P = 0.016$); for *Shigella* or enteroinvasive *E. coli*, 3.70 (95% CI: 1.38–9.92; $P = 0.009$) and for *Campylobacter*, 4.72 (95% CI: 1.68–13.27; $P = 0.003$; Fig. 1). Of viruses, rotavirus had an odds ratio for diarrhea of 2.33 (95% CI: 1.02–5.33; $P = 0.046$) and sapovirus of 8.46 (95% CI: 3.00–32.58;

$P = 0.002$). Of parasites, only *Cryptosporidium* correlated with diarrhea (odds ratio 5.96; 95% CI: 1.66–21.40; $P = 0.006$).

In quantitative analysis of reactive samples, we found a significant difference only for *Giardia*: children with diarrhea had lower median C_t values (IQR) than those without diarrhea [32 (5) vs. 38 (6); $P = 0.028$]. In case of enteropathogenic *E. coli*, only children with diarrhea had C_t below the cutoff value of 19.5 (7/53 vs. 0/48; $P = 0.013$).

DISCUSSION

In our study, multiplex PCR detected enteropathogens in stool samples of almost all children, whether with diarrhea (97%) or not (84%). In Rwandan children, the detection rate of PCR was equal, 94% in diarrhea patients and 79% in controls.²⁴ In China, PCR for 10 bacterial and viral pathogens was positive in 82% of children with diarrhea and in 47% of control children.¹³ The GEMS, which used mostly traditional methods, identified at least one putative pathogen in 83% of children with moderate to severe diarrhea and in 72% of controls.¹¹ In a cohort study in 8 sites in the developing world (MAL-ED), where mostly traditional testing was used, at least 1 pathogen was detected in 77% of diarrheal stools and 65% of non-diarrheal stools of children 0–24 months of age.¹²

In our study, PCR identified more pathogens, bacteria or viruses in diarrhea than in solid stools. If a specimen was positive, more than 1 pathogen was detected in diarrhea in 87% and in 59% in non-diarrhea. In Rwanda, the corresponding rates for the PCR-positive cases were 67% and 67%.²⁴ Two or more agents were identified in 53% of the diarrhea cases versus in 43% of controls in the GEMS¹¹ and in 53% and 45% in the MAL-ED study,¹² respectively. In Bengo province, Angola, of traditionally tested diarrhea samples, only 37% of the positive samples had 2 or more pathogens identified.³ There is no doubt that the modern methodology vastly improves detection of possible enteropathogens.

In our study, diarrhea correlated with the detection of enterotoxigenic and enteroaggregative *E. coli*, *Shigella* or enteroinvasive *E. coli*, *Campylobacter*, rotavirus, sapovirus and *Cryptosporidium*.

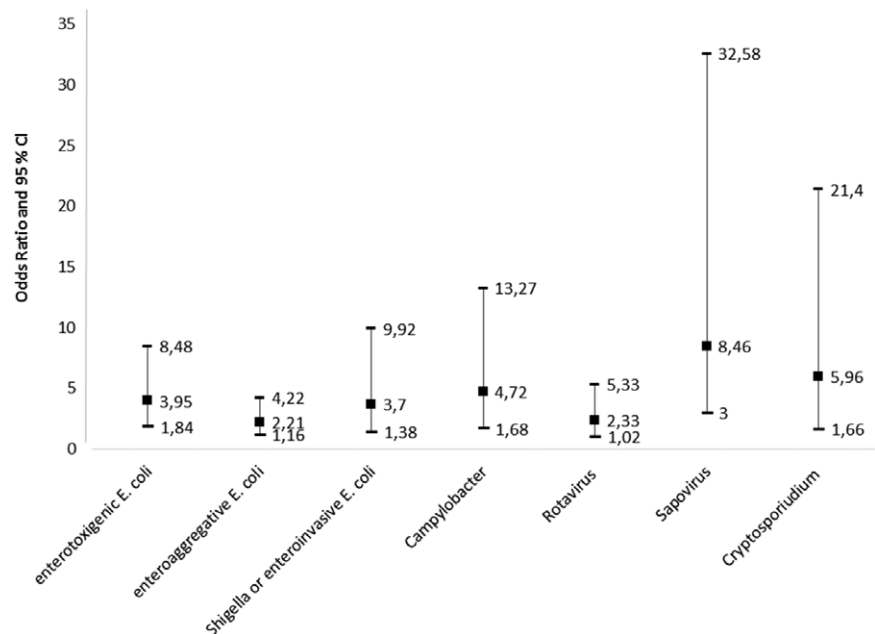


FIGURE 1. Odds ratios and 95% confidence intervals for diarrhea of pathogens with significant results.

In Rwanda, enterotoxigenic *E. coli*, *Shigella*, rotavirus and, contrary to Luanda, also norovirus were associated with symptomatic infection.²⁴ Unlike our study, in the GEMS study, enteroaggregative *E. coli* did not correlate with diarrhea but enteropathogenic *E. coli* did.¹¹ In our study, diarrhea correlated with high quantity of enteropathogenic *E. coli*. In the MAL-ED study, *Campylobacter*, rotavirus and norovirus associated with diarrhea in children 0–24 months of age, *Cryptosporidium* only in the first year of life and *Shigella* and astrovirus in the second year of life.¹² In our study, the number of detected pathogens increased with age in children with diarrhea; in the MAL-ED study, this correlation was seen in all children, especially during the first year of life.¹²

In the multicenter study in which 15 enteropathogens were searched for and the analytical cutoff was set at 35 quantification cycles, *Campylobacter*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, *Shigella*/enteroinvasive *E. coli*, *Vibrio cholerae*, rotavirus and *Cryptosporidium* associated with diarrhea, and the association was clearer at high pathogen loads.¹⁷ Of interest, *Giardia* was more common in control children,¹⁷ whereas in our study, diarrhea correlated with high *Giardia* load. In Tanzanian children with quantitative detection with TaqMan array cards, no pathogens were significantly associated with diarrhea. However, when pathogen quantity was taken into account, an association was observed for *Shigella*/EIEC, rotavirus and astrovirus.²⁵ Here, sapovirus was associated with diarrhea, as was the case in India for the 24- to 59-month-old children.¹¹ Again, rather dissimilar findings were observed in Rwanda and Liu's multicenter study, where sapovirus was more common in control children.^{17,24}

Our study has limitations. The number of the samples examined was restricted because of limited resources. The children without diarrhea were older than children with diarrhea. Therefore, we tested the relevance of different pathogens causing diarrhea, taking into account age as confounding variable. The stool samples of children without diarrhea were collected later in the year than from those with diarrhea. We do not, however, think this skewed the results because the findings remained rather constant throughout all months of the year. All clinical data were not recorded, but we believe this happened randomly and did, thus, not distort the results. Differences in case definitions can make between-study

comparisons difficult. For example, the present study, and the MAL-ED study,¹² included all children with diarrhea (3 or more loose stools or at least 1 bloody stool a day), while in the GEMS study, the episode had to be moderate to severe.¹¹

We detected potential enteropathogens more than has been the case in most other studies. Also, here, more than 1 pathogen was detected with high frequency. Even if Angola is considered an upper middle-income country, wealth is distributed unevenly, and most children served by the Pediatric Hospital represent poorer population. In this kind of low-resource settings with overcrowding and poor hygiene, the children are repeatedly exposed to multiple pathogens.

In a setting in which several potential pathogens are detected in the same sample, and the same putative pathogens are found also in children without diarrhea, the role of each single pathogen in terms of causing diarrhea is difficult to determine. PCR has high sensitivity and can detect pathogens in low quantities with unclear significance, whereas quantitative PCR may help to distinguish clinically important infection from asymptomatic carriage.^{25,26} It is likely that the load of each pathogen is important. It is also thought that subclinical infections may cause physiologic and structural alterations of the gut, which lead to repeated episodes of diarrhea, impaired gut function and growth impairment.^{12,29}

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